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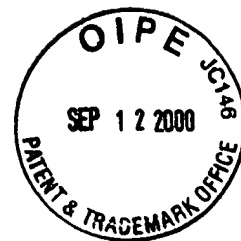
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Slit Proteins Bind Robo Receptors and Have an Evolutionarily Conserved Role in Repulsive Axon Guidance

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Summary

Extending axons in the developing nervous system are guided in part by repulsive cues. Genetic analysis in *Drosophila*, reported in a companion to this paper, identifies the Slit protein as a candidate ligand for the repulsive guidance receptor Roundabout (Robo). Here we describe the characterization of three mammalian Slit homologs and show that the *Drosophila* Slit protein and at least one of the mammalian Slit proteins, Slit2, are proteolytically processed and show specific, high-affinity binding to Robo proteins. Furthermore, recombinant Slit2 can repel embryonic spinal motor axons in cell culture. These results support the hypothesis that Slit proteins have an evolutionarily conserved role in axon guidance as repulsive ligands for Robo receptors.

Introduction

The ventral midline of the nervous systems of both vertebrate and invertebrate organisms acts as an important intermediate target for axons navigating along distinct trajectories. Embryological experiments and genetic manipulations have suggested that growth cones sense specific cues at the midline that influence their decision to cross or not to cross, but the identity of these midline cues and the precise molecular mechanisms mediating this choice are only now starting to be identified (reviewed in Tessier-Lavigne and Goodman, 1996).

At the ventral midline, an axon's decision whether to cross appears to be determined by a finely regulated balance between both attractive and repulsive guidance cues and the axon's changing responsiveness to these signals. Just as netrin proteins have been implicated in attracting axons to the midline in nematodes, insects,

and vertebrates, midline cells also appear to express counterbalancing inhibitory cues that push axons away (reviewed in Tessier-Lavigne and Goodman, 1996). For instance, in vertebrates, ablation of the ventral midline, either surgically or genetically, results in a disruption of axon trajectories such that a proportion of axons that would normally cross now fail to and those that would normally not cross, now do so aberrantly (reviewed in Colamarino and Tessier-Lavigne, 1995). Experiments in chick embryos have provided further evidence that the ventral midline floor plate cells express a contact-dependent repellent cue whose activity is normally masked by the attractive cell adhesion molecule NrCAM (Stoeckli et al., 1997). In addition to being a source of attractants and repellents, the ventral midline is also able to alter an axon's ability to respond to these cues. For instance, upon crossing the floor plate, axons that were responsive to the floor plate-derived chemoattractant Netrin 1 prior to crossing are, after crossing, no longer able to respond to this cue (Shirasaki et al., 1998).

In *Drosophila*, the midline also appears to control growth cone properties. Thus, the Roundabout (Robo) receptor is downregulated on crossing axons at the midline, by a mechanism involving the Commissureless protein. After crossing the midline, Robo is again specifically upregulated, thus ensuring that these axons do not recross again (Seeger et al., 1993; Tear et al., 1996; Kidd et al., 1998a, 1998b). In *robo* loss-of-function mutants, axons cross and recross the midline inappropriately. Robo is highly conserved across species, both in sequence and apparent function (Kidd et al., 1998a; Zallen et al., 1998). In particular, in the rat spinal cord, Robo is expressed in a pattern consistent with a role in mediating guidance decisions at the floor plate (Kidd et al., 1998a).

In a companion paper, genetic evidence is provided that the secreted extracellular matrix molecule Slit is a ligand for *Drosophila* Robo (Kidd et al., 1999 [this issue of *Cell*]). *Drosophila* Slit protein is expressed by midline glia and appears to accumulate on axons (Rothberg et al., 1990). In *slit* mutant embryos, axons that would either normally not cross or recross the midline, now enter the midline and remain there. *slit* and *robo* display dosage-sensitive interactions, suggesting that these molecules are in a common molecular pathway (Kidd et al., 1999). In this paper we present the cloning of mammalian homologs of *slit* and show that, like their *Drosophila* counterpart, vertebrate Slit genes are expressed by cells at the ventral midline of the nervous system. We further demonstrate that Slit proteins are ligands for Robo proteins in both *Drosophila* and vertebrates and that in vertebrates Slit2 can repel spinal motor axons in culture. Together with genetic data (Kidd et al., 1999), these results establish that Slit is a repulsive ligand for Robo in *Drosophila* and that Slit proteins have a conserved function in repulsive axon guidance. Similar results on vertebrate Slit proteins have been obtained by Li et al. (1999 [this issue of *Cell*]).

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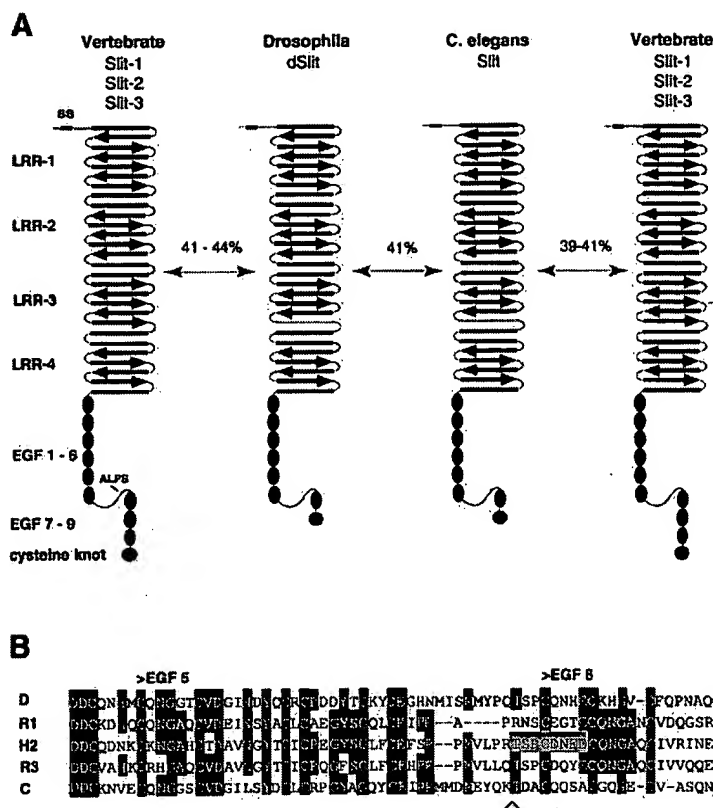


Figure 1. Domain Structures and Homologies of Slit Family Members

(A) Diagram illustrating the domain structures of Slit family members and percent amino acid identities among these members. All identified Slit proteins contain signal peptide (ss), four tandem leucine-rich repeats (LRR), EGF repeats, a conserved ALPS spacer, and a cysteine knot. Both *Drosophila* Slit and *C. elegans* Slit lack one LRR in LRR-3 that is present in the three mammalian Slit proteins, and they contain seven EGF repeats compared to nine EGF repeats in the mammalian proteins.

(B) Amino acid sequences corresponding to EGF5 and part of EGF6 of *Drosophila* Slit (D), rat Slit1 (R1), human Slit2 (H2), rat Slit3 (R3), and *C. elegans* Slit (C) are shown to illustrate conservation of the putative proteolytic cleavage site. The peptide sequence obtained by microsequencing the amino terminus of the C-terminal cleavage fragment of hSlit2 (Slit2-C) is shown shaded, and the putative cleavage site in hSlit2 is marked by a triangle.

Results

Evolutionary Conservation of Slit Proteins

Previous studies in *Drosophila* identified Robo (or dRobo1) as a repulsive guidance receptor on growth cones that keeps navigating axons from crossing the midline inappropriately (Kidd et al., 1998a). In a companion paper, Kidd et al. (1999) provide genetic evidence implicating Slit as a ligand for dRobo1. Given the high sequence conservation between vertebrate and invertebrate Robo family members (Kidd et al., 1998a), we reasoned that a Slit homolog(s) was likely also to be a ligand for vertebrate Robo proteins. We identified several human and mouse expressed sequence tags that exhibited high homology to dSlit and used these to probe both a human fetal brain library and an embryonic day 13 (E13) rat spinal cord library (see Experimental Procedures). The clones recovered corresponded to three distinct genes, all with high amino acid identity to dSlit. We designated the human and rat cDNAs *hSlit1*, -2, and -3, and *rSlit1*, -2, and -3, respectively (Figure 1; see Experimental Procedures for sequence information). Recently, three other groups have also reported the cloning of human (*hSlit1/MEGF4*, *hSlit2*, and *hSlit3/MEGF5*) and rat (*MEGF4* and *MEGF5*) slit family members (Itoh et al., 1998; Holmes et al., 1998; Nakayama et al., 1998). We have chosen to adopt the nomenclature of Itoh et al., 1998, for clarity. All mammalian Slit proteins share a common domain structure and high sequence homology with *Drosophila* Slit (43.5%, 44.3%, and 41.1% between dSlit and Slit1, -2, and -3, respectively), as well

as with one another (60%–66% overall) (Figure 1A). All of the predicted Slit proteins contain a putative signal peptide, four tandem arrays of leucine rich repeats (LRRs) (which are flanked by conserved amino and carboxy-terminal sequences), a long stretch of EGF repeats, an Agrin-Laminin-Perlecan-Slit (ALPS) conserved spacer motif, and a cysteine knot (a dimerization motif found in several secreted growth factors). Like their *Drosophila* counterparts, the mammalian Slit proteins lack any hydrophobic sequences that might indicate a transmembrane domain and are thus predicted to encode secreted extracellular proteins. These conserved motifs are also found in a number of other proteins and have been implicated in mediating protein–protein and ECM–protein interactions (reviewed in Iozzo, 1997; Rothberg et al., 1990, 1992). Slit1, -2, and -3 differ from *Drosophila* Slit in that they contain an additional LRR in the third tandem LRR array and two additional EGF repeats (Figure 1A). A previously published *Drosophila* Slit sequence (Rothberg et al., 1990) lacks an LRR in the first tandem LRR array that is present in vertebrate Slits and in a novel *Drosophila* Slit sequence isolated by Kidd et al. (1999). Interestingly, the *C. elegans* Slit homolog (Figure 1A; Itoh et al., 1998), like dSlit, also contains this additional LRR in LRR-1, has only seven EGF repeats, and is also lacking the extra LRR found in LRR-3 in vertebrates. This suggests that the addition of the EGF repeats occurred after the divergence of the chordate lineage from arthropods and nematodes during metazoan evolution but prior to the triplication of the ancestral *slit* gene in the vertebrate lineage.

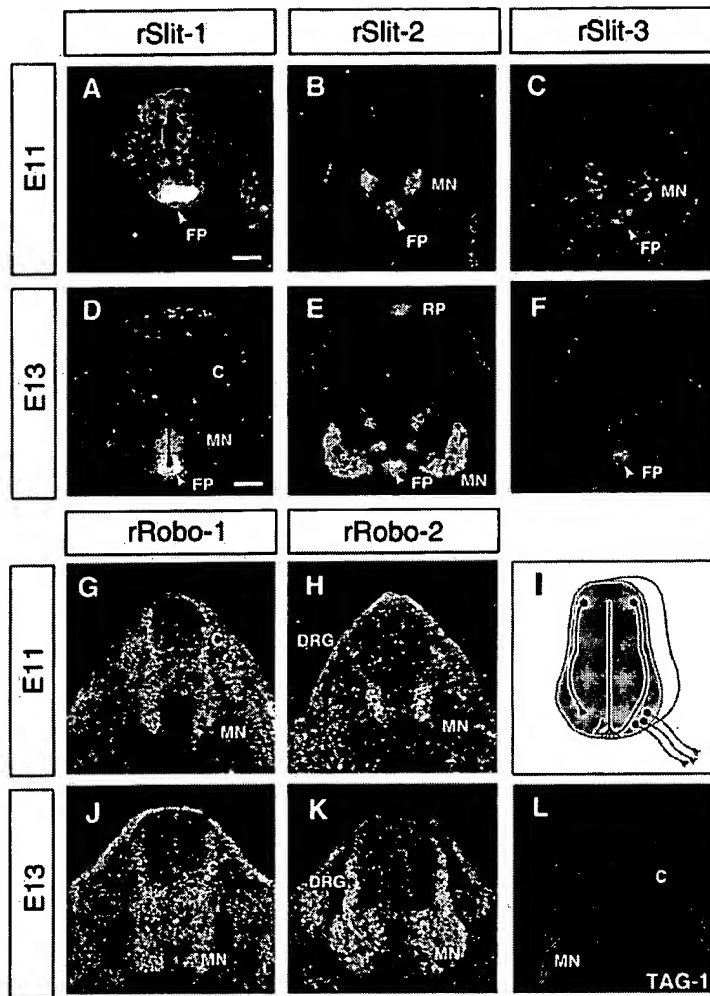


Figure 2. Expression of *Slit1*, -2, and -3, and *Robo1* and -2 in the Developing Rat Spinal Cord

Expression of *Slit1*, *Slit2*, and *Slit3* at E11 (A-C) and E13 (D-F) and *Robo1* and *Robo2* at E11 and E13 (G, H, J, and K) in transverse sections of the rat spinal cord. Semiadjacent sections from forelimb level were used at each age.

(A and D) At E11 and E13, *rSlit1* is expressed at high levels in the region of the floor plate (FP, yellow arrowhead), with lower levels of expression in the intermediate and dorsal ventricular zone (E11) and in the regions of the commissural (C), association, and motor neuron (MN) cell bodies (E13).

(B and E) At E11 and E13, *rSlit2* is expressed by the floor plate, by the roof plate (RP), and in the motor column (MN; E11 and E13), where at E13 it appears to be expressed by specific subpopulations (note the apparent holes in expression, red arrowheads). Although the E11 section shown lacks roof plate expression, this was not consistent for all E11 sections. Whether this indicates heterogeneity in roof plate expression along the rostro-caudal axis or a sectioning artifact is unclear.

(C and F) At E11 and E13, *rSlit3* is expressed by the floor plate but at significantly lower levels than *rSlit1* or *rSlit2*. *rSlit3* is also expressed transiently from E11 (C) to E12 (data not shown) in the motor column, disappearing by E13 (F).

(G-L) As previously reported (Kidd et al., 1998a), from E11 (G) to E13 (I) *rRobo1* is expressed dorsally in the region of the commissural and association neuron cell bodies and ventrally in subpopulations of motor neurons. *Robo2* is expressed in the motor column, but in a pattern distinct from *Robo1*, in the dorsal root ganglia (DRG), and dorso-laterally along the edge of the spinal cord (H and K). Whether this lateral expression corresponds to neurons or to other cell types is unclear. (I and L) Schematic and TAG-1 staining of commissural and motor axon trajectories at E13.

Scale bars, 50 μ m (A-C, G, and H) and 100 μ m (D-F and J-L).

Mammalian *Slit* Genes Are Expressed in Overlapping Regions of the Developing Spinal Cord

We next determined the expression of *Slit1*, *Slit2*, and *Slit3* mRNAs in comparison to *Robo1* and *Robo2* mRNAs by in situ hybridization in the developing rat spinal cord at embryonic stages E11-E13, i.e., when commissural axons are migrating to the midline (Altman and Bayer, 1984). As reported previously (Kidd et al., 1998a), rat *Robo1* expression overlaps with that of *Dcc* and TAG-1, known markers for commissural (dorsal) and motor (ventral) neurons at these stages (data not shown, Figures 2G, 2J, and 2L). Beginning at E11, rat *Robo2* is also expressed ventrally in the region of the developing motoneuron cell bodies, but in a pattern distinct from that of rat *Robo1*. In contrast to *rRobo1*, *rRobo2* is expressed in the dorsal root ganglia (DRG) by E13 (Figures 2H and 2K) but is not detected in the region of the commissural neurons overlapping with *Dcc* and TAG-1 (Figure 2K). Expression of *rRobo2* is, however, detected dorsally in a lateral region of the spinal cord that may comprise the

cell bodies of distinct subpopulations of commissural or association neurons (Figure 2K).

Like *Drosophila slit*, which is expressed by cells at the midline of the *Drosophila* nervous system, the mRNAs for all three rat *Slit* proteins are expressed by floor plate cells at the ventral midline of the spinal cord (Figures 2A-2F). From E11-E13, *Slit1* is expressed at high levels in the floor plate and at lower levels in other regions of the spinal cord, including in the region of commissural and association neurons and in the motor column (Figures 2A and 2D). Likewise, *Slit2* is also expressed by the floor plate, but in a different pattern: whereas *Slit1* is expressed broadly by both floor plate cells and cells in the ventral portion of the ventricular zone, *Slit2* is restricted to the most basal and medial region of the floor plate. *Slit2* is expressed at high levels in the developing motor column and the roof plate, as well as very weakly in the region of the commissural neurons in the dorsal spinal cord (Figures 2B and 2E). Like *Slit1* and -2, *Slit3* is also expressed by the floor

plate but at significantly lower levels than *Slit1* and *Slit2* (Figure 2C and 2F). In addition, whereas the spatial distributions of *Slit1* and *Slit2* in the spinal cord remain largely constant between E11 and E13, *Slit3* expression is dynamic: at E11 and E12 *Slit3* is expressed in the motor column, but by E13, the stage at which most commissural axons have crossed the floor plate and have turned rostrally, *Slit3* expression in the motor column is significantly diminished and expression is largely restricted to the floor plate. These results are in general agreement with the expression studies of Holmes et al. (1998) and Itoh et al. (1998). It is striking that, in addition to having specific additional sites of expression, the three mammalian *Slit* genes, like their counterpart in *Drosophila*, are all expressed by cells at the ventral midline of the nervous system. This expression is consistent with a potential role for *Slit* proteins in repulsive axon guidance at the midline mediated by Robo proteins.

Slit Is Proteolytically Processed in Cell Culture and In Vivo

As a first step toward testing whether *Slit* proteins are ligands for Robo proteins, we sought to generate recombinant *Drosophila* and mammalian *Slit* proteins by expressing epitope-tagged constructs in cultured cells. Because human *Slit2* was the first *Slit* gene for which we obtained a full-length cDNA sequence, we focused on its protein product. hSlit2 expression constructs bearing either a FLAG tag at the amino terminus or a myc tag at the carboxyl terminus were transiently transfected into COS and 293T cells, and expression was determined by immunohistochemistry and Western blot analysis. hSlit2 protein can be detected on the surface of living cells with an antibody directed against either the N- or C-terminal tags, indicating that hSlit2 is secreted but remains associated with cell surfaces (Figure 3C and data not shown). Western blots of conditioned media and high salt (1 M NaCl) extracts of membranes from transfected cells revealed a band migrating at 190 kDa, which is slightly higher than the predicted size for hSlit2 and presumably reflects a glycosylated form of the protein (Wang et al., 1999 [this issue of *Cell*]). In addition to this 190 kDa isoform, we also detected two additional bands: a 140 kDa protein that comprises the amino terminus of hSlit2 (as detected with an antibody against the N-terminal FLAG tag) and a ~55–60 kDa protein that comprises the carboxyl terminus (as detected with an antibody against the C-terminal myc tag) (Figures 3A and 3B). Since the molecular masses of these two proteins roughly add up to that of full-length hSlit2, we presume that they arise from proteolytic cleavage of the full-length protein. The amino- and carboxy-terminal fragments will be referred to as Slit2-N and Slit2-C, respectively. Expression of hSlit2 in a number of other cell lines, including 293-EBNA and NIH-3T3, resulted in identical patterns of cleavage, as assessed by Western blotting (data not shown). In fact, this cleavage was already predicted since we had purified a 140 kDa amino-terminal fragment of *Slit2* from bovine brain (Wang et al., 1999). These results also indicate that the cleavage observed in vitro also occurs in vivo in mammals.

Full-length Slit2, Slit2-N, and Slit2-C exhibit different cell association characteristics. The majority (>90%) of the 190 kDa full-length hSlit2 was found associated with

cell surfaces (Figure 3 and data not shown) but could be readily extracted from membranes with either high salt (1 M NaCl) or heparin, suggesting its association with cell surfaces via heparan sulfate proteoglycans or other negatively charged moieties. hSlit2-C was more diffusible, partitioning roughly equally between the conditioned medium and cell surfaces. In contrast, hSlit2-N was largely absent from the conditioned medium and was found to be tightly cell associated and more resistant to heparin extraction, requiring several consecutive high salt washes to be fully released (Figures 3A and 3B).

Rothberg et al. previously reported that *Drosophila* S2 cells endogenously express dSlit that can be detected in both the culture medium and extracellular matrix material deposited by the S2 cells. They did not, however, report any evidence for cleavage of the full-length ~190 kDa *Slit* protein (Rothberg et al., 1990; Figure 3D). We have verified this original observation and extended it by showing that expression of a recombinant version of *Drosophila* *Slit* tagged at its carboxyl terminus with alkaline phosphatase (*Slit*-AP) resulted in the generation of only the full-length protein (Figure 3D). To determine whether this is due to differences between the *Drosophila* and human proteins (e.g., the possible absence of a cleavage motif in *Drosophila* *Slit*) or to differences in the expression systems, we expressed *Drosophila* *Slit*, either untagged or bearing a C-terminal AP tag, in 293T and COS cells and examined its processing by immunoblotting with an antibody against the carboxy-terminus of dSlit. As observed for hSlit2, bands of ~190 kDa and ~55–60 kDa were detected (data not shown), the latter presumably representing a carboxy-terminal cleavage fragment of *Slit*, *Slit*-C (Figure 3D; the N-terminal fragment was not tagged and so could not be detected in these experiments).

Taken together, these results suggest that mammalian tissue culture cells contain one or more *Slit*-cleaving proteases which appear to be absent from or inactive in S2 cells. As observed for the mammalian protein (Wang et al., 1999), this processing of dSlit also seems to occur in vivo, as the antibody against dSlit detects two similarly sized bands of ~190 kDa and ~55–60 kDa on Western blots of *Drosophila* embryo extracts (Figure 3C).

To begin to identify the cleavage site, we purified recombinant Slit2-C (see Experimental Procedures) and microsequenced its amino terminus by Edman degradation. The resulting sequence, TSPCDNFD, is found at the beginning of the 6th EGF repeat of hSlit2 (Figure 1B) and is consistent with the peptide sequences derived from microsequencing of the amino terminal fragment of bovine *Slit2* purified from calf brain (Wang et al., 1999), all of which mapped to regions amino-terminal to this site. We cannot, however, exclude that the actual cleavage site is located slightly more amino-terminal and that the true amino terminus of Slit2-C undergoes further cleavage or degradation. This sequence is at least partially conserved among *Drosophila* and mammalian *Slit* family members (Figure 1B), suggesting that the cleavage site may also be conserved between insects and vertebrates. This sequence is not, however, well conserved in the *C. elegans* *Slit* homolog (Figure 1B).

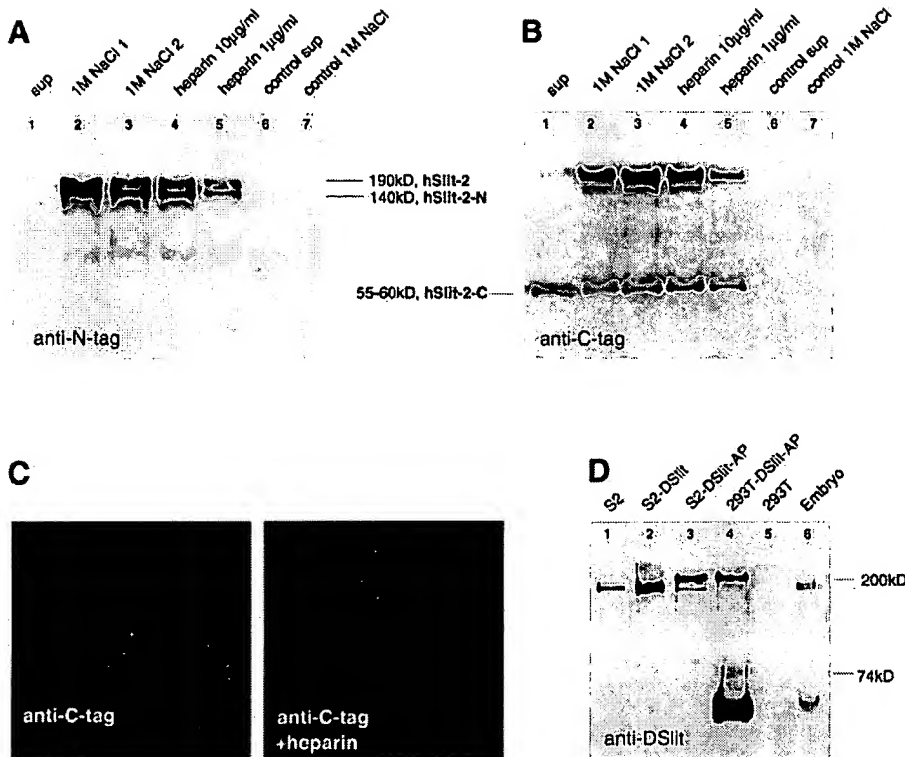


Figure 3. Human Slit2 and *Drosophila* Slit are Proteolytically Processed and the Cleaved Fragments Have Different Cell Association Characteristics

(A and B) Full-length hSlit2 (190 kDa) expressed in 293T cells is processed into a 140 kDa N-terminal fragment (detected by an antibody against an N-terminal FLAG-tag; A) and an ~55–60 kDa C-terminal fragment (as detected by an antibody against a C-terminal myc tag; B). On silver stained gels, this band appears as a doublet, migrating at 55 and 60 kDa, both of which correspond to fragments of hSlit2 (as assessed by Edman degradation sequencing) and thus presumably represent different glycosylation states.

(A–C) The three cleavage fragments associate with cell surfaces to different extents. hSlit2-C is diffusible and can be detected in conditioned medium (B, lane 1). Full-length hSlit2 and hSlit2-N are tightly cell associated, accumulating at low or undetectable levels in the conditioned medium (compare lanes 1, A and B) and require multiple extractions with 1 M NaCl for full extraction from cell surfaces (A and B, lanes 2 and 3). All three fragments can be removed to some extent from cell surfaces by heparin, as seen by Western blotting (A and B, lane 4) or immunohistochemistry (C).

(D) *Drosophila* Slit is processed when expressed in mammalian cells but not S2 cells. S2 cells, which endogenously express moderate levels of dSlit (lane 1), when transfected with untagged dSlit (lane 2) or AP-tagged dSlit (lane 3) expression constructs express an ~190 kDa protein, corresponding to dSlit, as detected by a mAb against the C terminus of dSlit (the lower mobility of dSlit-AP is due to the presence of the AP tag). 293T do not express any endogenous proteins that cross-react with the anti-dSlit mAb (lane 5). When expressed in 293T cells, the 190 kDa dSlit (minor species) is processed to give a 55–60 kDa presumed C-terminal fragment (lane 4). The 140 kDa N-terminal fragment is presumably present but is not detected with this antibody. Bands of similar sizes can also be detected on blots of *Drosophila* embryo extracts (lane 6).

Conserved Binding of Robo Proteins to Slit Proteins

The availability of expressed Slit proteins enabled us to examine their interactions with Robo proteins in a cell overlay binding assay. Media conditioned from cells transfected with a C-terminally tagged hSlit2 expression construct was applied to COS cells expressing either recombinant rat Robo1 or Robo2 and binding was detected with an antibody against the myc tag. Conditioned medium was prepared by extracting hSlit2-expressing cells with 1–10 µg/ml heparin and therefore consisted of a mixed population of N-terminal, C-terminal, and full-length hSlit2 isoforms (Figure 3B). Cells expressing either rRobo1 or rRobo2 showed significant binding of hSlit2 (Figures 4A and 4B). Similarly, *Drosophila* Slit, applied in supernatants from transfected S2 cells, was found to bind COS cells expressing dRobo1, as detected with the antibody against the C terminus of

dSlit (Figure 4G). In these assays, only 10%–20% of transfected COS cells expressed tagged rRobo1 and rRobo2, with roughly equal levels of expression (as measured by number of expressing cells and intensity of associated fluorescence) for rRobo1 and rRobo2. Even fewer cells were found to express dRobo on their surfaces (data not shown). Binding assays were performed in the presence of heparin, which reduces the nonspecific background binding of Slit to cell surfaces (data not shown) but does not reduce binding to cells expressing Robo. The binding of Slit and Robo proteins is specific, as hSlit2 and dSlit did not bind cells expressing DCC, TAG-1, or L1 (Figures 4D and 4H and data not shown), other members of the immunoglobulin superfamily which, like Robo genes, are also expressed by commissural and motor neurons (Moos et al., 1988; Furley et al., 1990; Keino-Masu et al., 1996).

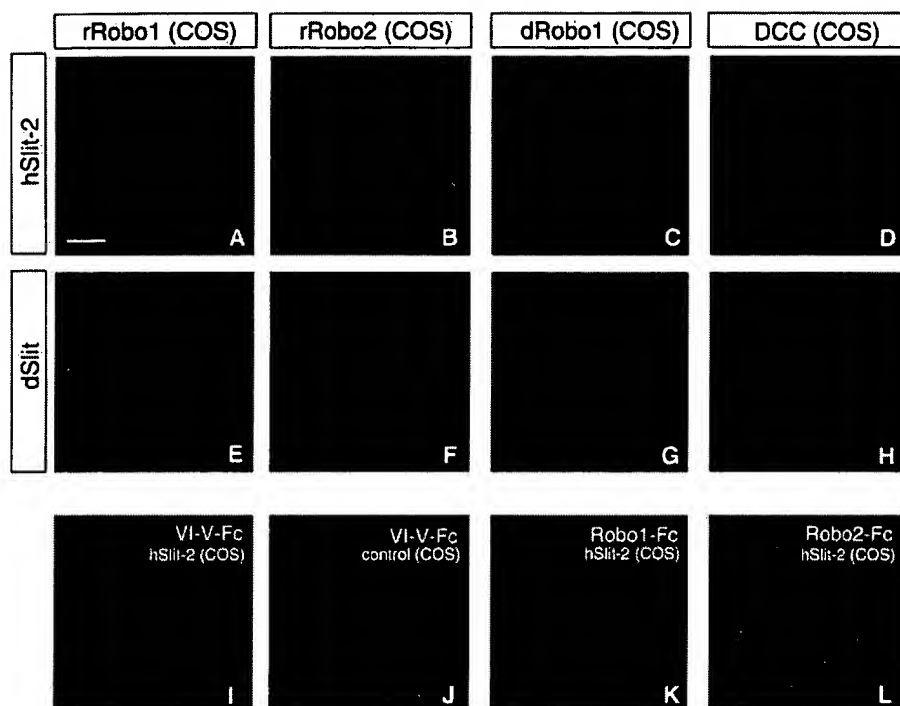


Figure 4. dSlit and hSlit2 Bind dRobo1, rRobo1, rRobo2, as well as Netrin 1

Supernatants from cells expressing hSlit2 (A-D) or dSlit (E-H) were incubated with cells expressing Robo proteins (A-C and E-G) or control members of the immunoglobulin superfamily, DCC (D and H), TAG-1, and L1 (data not shown) in the presence of 2 μ g/ml heparin (which reduces background binding). Binding was detected using an antibody against the C-terminal myc tag on hSlit2 (A-D) or an antibody against the C terminus of dSlit (E-H) and corresponding Cy3-conjugated secondary antibodies. hSlit2 binds rRobo1 (A), rRobo2 (B), as well as dRobo1 (C), but not DCC (D), TAG-1, or L1 (data not shown). dSlit binds dRobo1 (G), as well as rRobo1 (E) and rRobo2 (F), but not DCC (H), TAG-1, or L1 (data not shown). In addition, soluble rRobo1-Fc and r-Robo2-Fc can bind cells expressing hSlit2 on their surfaces (K and L). Netrin 1 (VI-V-Fc), binds cells expressing hSlit2 (I) but not control cells (J). Scale bar, 100 μ m.

The interaction between Slit and Robo proteins was further demonstrated using soluble forms of the ectodomains of rRobo1, rRobo2, and dRobo1 fused to either the constant region (Fc) of the human immunoglobulin molecule or to alkaline phosphatase. We examined whether these proteins could bind cells expressing various Slit proteins on their surfaces. rRobo1-Fc and rRobo2-Fc bound transfected cells expressing hSlit2, and dRobo-AP bound cells expressing dSlit (Figures 4K and 4L). In control experiments, we found that Robo1-Fc and Robo2-Fc did not bind cells expressing either F-spondin or Netrin 1, both extracellular matrix proteins made by floor plate cells, nor did they bind cells expressing Semaphorin III (data not shown). Like Slit proteins, all of these proteins associate with cell membranes (Klar et al., 1992; Luo et al., 1993; Serafini et al., 1994).

These results indicate a high degree of specificity in the interaction between Slit and Robo proteins. In addition, in cross-species experiments we found that the binding interactions are evolutionarily conserved. Thus, dSlit bound cells expressing either rRobo1 or rRobo2, and hSlit2 bound cells expressing dRobo1, although these interactions appeared weaker than those observed within species (Figures 4C, 4E, and 4F). Similarly, Robo1-Fc and Robo2-Fc bound cells expressing dSlit, and dRobo-AP bound hSlit2-expressing cells, as well as dSlit attached to protein A beads (data not shown).

The amino-terminal LRRs of *Drosophila* and mammalian Slit proteins have homology to a number of ECM molecules, including the laminin-binding molecule biglycan (Rothberg et al., 1990). This prompted us to ask whether Slit proteins can also bind laminins. We found that hSlit2, applied in conditioned medium from transfected cells, bound to a substrate with laminin 1, but not a substrate coated with fibronectin (data not shown). Since Netrin proteins show homology to a portion of the laminin molecule (Serafini et al., 1994), we examined whether Netrin 1 and Slit2 can bind one another. For this, we used a soluble, truncated but bioactive form of Netrin 1, in which the two amino-terminal domains of Netrin 1 (domains VI and V) are fused to Fc (Keino-Masu et al., 1996). This Netrin 1 (VI-V-Fc) protein bound to COS cells expressing hSlit2, but not to mock transfected cells, in a pattern that was indistinguishable from binding observed with Robo1-Fc and Robo2-Fc (Figure 4I).

The binding affinities for the interactions between Robo proteins and Slit proteins, and between Slit proteins and Netrin 1, were estimated in equilibrium binding experiments. Robo-Fc fusion proteins were used as binding probes on COS cells expressing hSlit2 or dSlit, or control COS cells. Binding of Netrin 1 (VI-V-Fc) was similarly tested on 293T cells expressing hSlit2, or control 293T cells. Specific binding of Robo1-Fc, Robo2-Fc, and Netrin 1 (VI-V-Fc) to hSlit2-expressing cells, and

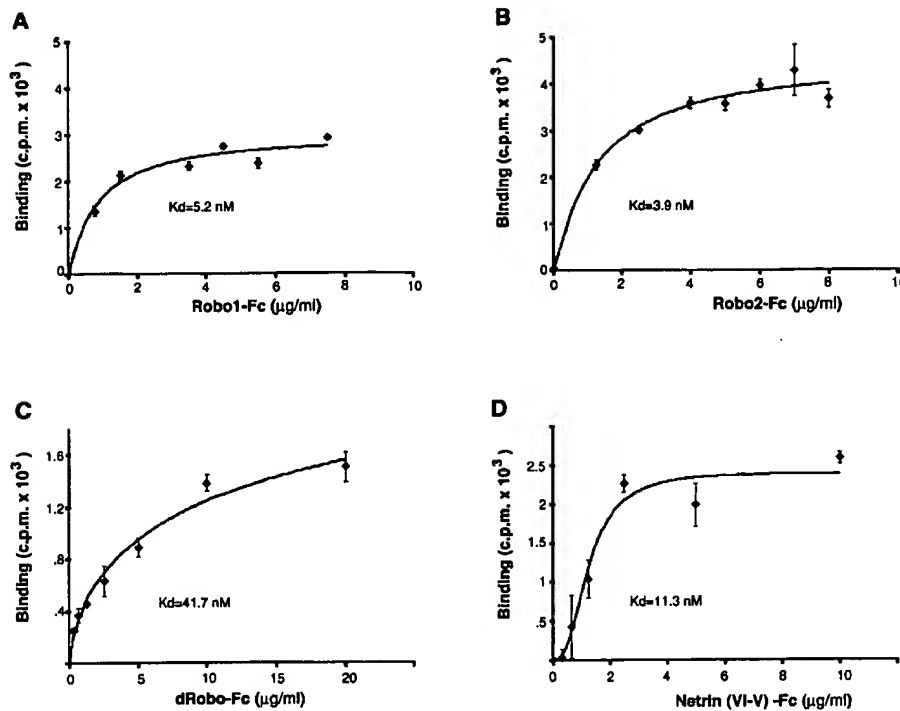


Figure 5. Equilibrium Binding Curves for Interactions of Robo, Slit, and Netrin Proteins

Equilibrium binding of rRobo1-Fc (A), rRobo2-Fc (B), Netrin 1(VI-V-Fc) (D) to hSlit2, and of dRobo-Fc to dSlit (C). COS (A–C) or 293T (D) cells transfected with either hSlit2 or dSlit expression constructs, or control vector alone, were incubated with indicated concentrations of purified rRobo1-Fc, rRobo2-Fc, dRobo-Fc, or Netrin 1(VI-V-Fc) in PBS/1%BSA for 4 hr, washed, incubated with 125 I-labeled anti-human IgG, and washed again. Total binding was determined by measuring radioactivity associated with cells after the final wash. Specific binding was defined as the difference between binding to Slit-expressing cells and vector-transfected cells. Specific binding curves were fitted using the Hill equation. K_d values for the interaction of hSlit2 with Robo1 (A), Robo2 (B), and Netrin 1(VI-V-Fc) (D) were 5.3 nM, 3.9 nM, and 11.3 nM, respectively, and the K_d for the interaction with dSlit with dRobo (C) was 41.7 nM. Results shown are from one of two representative experiments performed for each interaction. Bars indicate SEM for triplicates.

of dRobo-Fc to dSlit-expressing cells, showed saturation, and binding curves were fitted to the Hill equation, yielding values for the K_d s of 5.3 nM, 3.9 nM, 11.3 nM, and 41.7 nM, respectively (Figure 5). The dissociation constants for the interactions of Robo1 and Robo2 with hSlit2 (~4–5 nM) fit roughly with the concentration of Slit2-N that gives a near saturating effect in the sensory axon elongation and branching assay (~1 nM; Wang et al., 1999) and are comparable to the dissociation constants for interactions between Netrin 1 and its receptors, DCC and UNC5, and between Sema III and its receptor Neuropilin 1 (Keino-Masu et al., 1996; Leonardo et al., 1997; He and Tessier-Lavigne, 1997). The binding affinity of dRobo-Fc to dSlit was approximately 10-fold lower than that of Robo1-Fc, Robo2-Fc, or Netrin 1(VI-V-Fc) for hSlit2, a finding that fits with the intensity of binding observed visually (Figure 4).

Slit2 Can Function as a Diffusible Chemorepellent for Motor Axons In Vitro

To test whether a mammalian Slit protein can act as a repellent, we cocultured aggregates of hSlit2-expressing cells either in contact with or at a distance from explants of ventral spinal cord from E11 rat embryos. Tissues were cultured in a matrix consisting of a mixture of collagen and matrigel (a partially purified extracellular

matrix containing collagen and laminin). In this environment, unlike in a collagen matrix (Ebens et al., 1996), motor axons grow out of the explants profusely, presumably stimulated by a component in matrigel. When ventral explants were cultured either at a distance (75–200 μ m) from (Figure 6A) or in contact with (Figure 6B) aggregates of hSlit2-expressing cells, a clear repulsion of motor axons was observed that was not seen in cultures with control COS cell aggregates (Figure 6, compare [A] and [B] to [C] and [D]). In many cultures, axons that were originally directed toward the COS cell aggregate appeared to turn away. In most experiments, cultures were supplemented with 50 ng/ml heparin, which we reasoned might promote the diffusion of Slit proteins over greater distances. Heparin alone had no effect on outgrowth, and clear, although somewhat weaker, repulsion was also seen in cultures without heparin added (data not shown).

Cultures from representative experiments were stained with an antibody against neurofilament to label axons, and the extent of outgrowth from the distal and proximal sides of the explant was quantified based on associated fluorescence (Figures 6E and 6F, see Experimental Procedures). While control explants had approximately equal outgrowth on their proximal and distal sides, the distal sides of explants cultured at a distance from

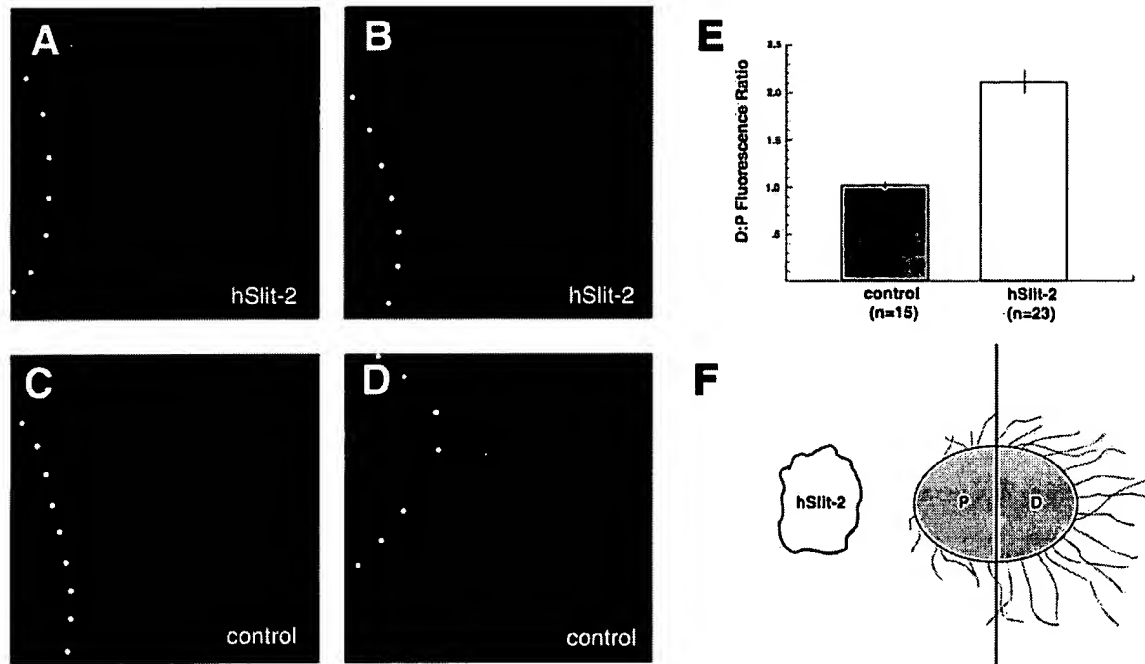


Figure 6. hSlit2 Can Repel Spinal Motor Axons

E11 ventral spinal cord explants were cultured either at a distance from (A and C) or in contact with (B and D) hSlit2-expressing or mock-transfected COS cells for 30 hr in a three-dimensional collagen gel matrix and then stained with an anti-neurofilament antibody to visualize axons. At this stage, only motor axons are expected to be found in these explants (Altman and Bayer, 1984; Ebens et al., 1996). Axons are repelled by COS cells expressing hSlit2 (A and B) but not mock-transfected COS cells (C and D). While repulsion appears to occur over greater distances when explants are in contact (compare A and B), clear repulsion of axons can also be seen when explants are placed 75–200 μ m apart (A). Explants from four separate experiments were photographed, digitized, and outgrowth from the proximal and distal sides of the explant was quantified based on associated fluorescence (F). A distal-proximal (D:P) fluorescence ratio was determined for each explant and averaged for each condition (E). While outgrowth in control cultures was largely radial (D:P ratio = $1.01 \pm .014$), outgrowth in hSlit2 cultures was biased away from the explant (D:P ratio = $2.10 \pm .146$) with, on average, 2-fold more outgrowth extending from the distal sides. Scale bar, 75 μ m.

hSlit2-expressing cells contained, on average, twice as much outgrowth as the proximal sides (Figure 6E). These results indicate that, at least under these culture conditions, hSlit2 can function as a diffusible repellent for developing motor axons.

Discussion

Genetic analysis has provided compelling evidence that in *Drosophila* the Slit protein is a repulsive ligand for the Robo receptor and prevents inappropriate midline crossing by Robo-expressing axons (Kidd et al., 1999). Here we verify a central prediction of this model by showing that Slit is indeed a ligand for Robo. We also provide evidence for a conservation of this repulsive guidance mechanism by showing that at least one vertebrate homolog of Slit—and presumably all three—can bind mammalian Robo proteins with high affinity and can repel at least one class of mammalian axons, spinal motor axons.

Slit Is a Ligand for the Robo Receptor in *Drosophila*
The *robo* gene was originally identified in a large-scale screen for genes controlling midline axon guidance (Seeger et al., 1993). In *robo* mutants, both crossing and

noncrossing populations of axons now cross the midline inappropriately (Seeger et al., 1993; Kidd et al., 1998a). This phenotype, together with the structure of Robo and its regulated expression on axons and growth cones, led to the hypothesis that Robo encodes a receptor for an unidentified midline repellent (Kidd et al., 1998a). Curiously, however, no other mutants recovered from the screen displayed a *robo*-like phenotype, as one might expect for mutants in a corresponding ligand.

The identification of the extracellular matrix molecule Slit as a candidate Robo ligand was precipitated by the finding that overexpression of *commisureless*, which results in loss of Robo protein expression, leads to a phenotype indistinguishable from that of loss of *slit* function (Kidd et al., 1999). In *slit* mutant embryos, growth cones that normally do not cross the midline, now enter the midline and never leave, despite the fact that they continue to express high levels of Robo. *slit* and *robo* display dosage-sensitive interactions, suggesting that they function in the same pathway, a suggestion supported by our demonstration that Slit is a Robo ligand. In addition to controlling the crossing of axons at the midline, dSlit is also required for the migration of muscle precursors away from the midline, suggesting that dSlit can act as both a long-range and short-range repellent

(Kidd et al., 1999). The discrepancy between the *robo* and *slit* mutant phenotypes suggests that Slit has binding partners in addition to Robo. The best candidate for this role is the closely related receptor dRobo2, which is also expressed by CNS neurons and can also bind Slit (J. Simpson, K. S. B., and C. S. G., unpublished results).

Conservation of the Biochemistry of Slit Proteins

Like other axon guidance molecules, Slit and its receptor Robo appear to be highly conserved through evolution. We and others have found that three vertebrate Slit proteins exhibit a high degree of sequence conservation with their *Drosophila* counterpart (~41%–44%) (Itoh et al., 1998; Holmes et al., 1998; Nakayama et al., 1998; Li et al., 1999). In addition, binding interactions of Slit and Robo proteins are also conserved, both within and across species. Interestingly, both hSlit2 and dSlit are proteolytically processed in mammalian tissue culture cells in an apparently similar way. We have also found evidence for similar processing in vivo of dSlit in *Drosophila* (this paper) and of Slit2 in bovine brain (Wang et al., 1999). We have not yet determined whether other Slit proteins are cleaved in this way, nor have we identified the protease(s) that mediates the cleavage. Candidates for the protease include metalloproteases (by analogy with the cleavage of the Notch ligand Delta by the metalloprotease Kuzbanian: Qi et al., 1998) or Furin-related proteases (by analogy with the cleavage of Semaphorin III by Furin: Adams et al., 1997). Since Slit proteolysis could in principle regulate and restrict Slit function, it will be of interest to determine whether the protease(s) acts intracellularly or extracellularly and where and when it is expressed.

Slit cleavage fragments appear to have distinct cell association characteristics, with the smaller C-terminal fragment being readily diffusible and the N-terminal and full-length fragments being more tightly cell associated. These different cell association characteristics suggest the possibility that processed fragments may also have different extents of diffusion and different binding interactions in vivo. It is also intriguing to speculate that the two fragments may also have different functional properties. Although the amino-terminal fragment of Slit2 alone appears capable of stimulating the elongation and branching of sensory axons in culture (Wang et al., 1999), it is not yet clear which fragment is responsible for the other activities documented for Slit proteins. In *Drosophila*, dSlit appears to be a short-range repellent for axons at the midline but a long-range repellent for mesodermal cells. This could be explained if the C-terminal fragment, which diffuses more readily, is responsible for long-range action, and the N-terminal fragment, which is more tightly cell associated in vitro, is responsible for the short-range action. Similarly, it is tempting to speculate that the C-terminal fragment of Slit2 is responsible for the long-range repulsive activity documented here on motor axons in vitro.

Another possibility, however, is that all of these other activities are explained by just one of the fragments, for instance the amino terminal fragment. According to this hypothesis, although the N-terminal fragment appears largely cell associated, a small amount that diffuses

away from cells would be sufficient to elicit the long-range effects. Such a model would require that in *Drosophila* mesodermal cells be capable of responding at lower concentrations than axons navigating the midline. A precedent for this is provided by the actions of Hedgehog proteins, which similarly undergo a proteolytic cleavage yielding a cell-associated N-terminal fragment and a freely soluble C-terminal fragment. These two fragments were initially suggested to mediate the distinct short- and long-range actions of Hedgehog proteins (Lee et al., 1994). Instead, it was found that both activities are mediated by the N-terminal fragment. The long-range actions appear to be explained by a low-level release from expressing cells and high sensitivity of responsive cells, whereas the short-range actions are mediated by mechanisms operating at higher threshold concentrations (reviewed in Bumcrot and McMahon, 1996). Whether the N-terminal fragments of Slit proteins mediate all of their activities and, similarly, which among the full-length Slit, N-terminal fragment, or C-terminal fragment of Slit are ligands for Robo proteins remain to be determined.

We have also found that Netrin 1 and hSlit2 interact with an affinity that is comparable to the affinities of hSlit2 for Robo proteins and of Netrin 1 for its high-affinity receptors. In addition, Slit1, -2 and -3 and Netrin 1 are coexpressed both in the floor plate and a number of locations throughout the nervous system, suggesting that this in vitro binding may reflect an in vivo interaction (Kennedy et al., 1994; Holmes et al., 1998; Ba-Charvet et al., 1999). For instance, binding of netrin and Slit proteins may either antagonize or potentiate the individual activities of these proteins. However, we have not yet found Netrin 1 to be able to either inhibit or to potentiate the repulsive effects of hSlit2 on motor axons (data not shown), nor does hSlit2 seem to be able to potentiate the outgrowth-promoting activity of Netrin 1 on spinal commissural neurons (data not shown). Another possibility is that a Slit-Netrin interaction functions in the localization of either or both of these proteins, a possibility that may be addressed by examining Slit protein localization in Netrin 1 knockout animals, and vice versa.

Slit and Robo and Midline Guidance

In the *Drosophila* CNS, dSlit is largely restricted to midline glial cells. Similarly, in the rat, all three Slit genes are expressed at the ventral midline of the developing spinal cord, consistent with a potential conserved role in guidance at the midline. Evidence for a conserved repulsive function of Slit proteins was obtained by demonstrating a repulsive effect of COS cells expressing hSlit2 on spinal motor axons extending from explants of ventral spinal cord in culture. This result is reminiscent of the finding that floor plate cells, a source of Slit2, can repel spinal motor neurons at a distance in a collagen gel coculture assay (Guthrie and Pini, 1995), raising the possibility that Slit2, or another Slit protein, may contribute to the chemorepulsive activity of floor plate cells.

While these results indicate that Slit2 can repel spinal motor axons in a contact-independent manner in vitro, it remains to be determined whether Slit proteins affect motor axon trajectories in a contact-dependent or -independent manner in vivo. Interestingly, in our in vitro

assay, the repulsive effect of hSlit2 usually appeared stronger when explant and cells were placed in contact than when they were separated (Figures 6A and 6B and data not shown). One explanation, which seems plausible given the cell association characteristics of hSlit2, is that this enhanced effect reflects differences in the ability of hSlit2 to diffuse in the collagen/matrigel matrix as opposed to the neurepithelium of explants. Matrigel is rich in laminin, which binds hSlit2, and thus diffusion of hSlit2 may be more restricted in this matrix than it is in the explant. As a result, individual growth cones may experience different concentration gradients of Slit protein depending on whether they are located in the explant or in the surrounding matrix. Even within the spinal cord explant, Slit diffusion is likely to be limited. For instance, a number of laminin isoforms are expressed in a restricted pattern by the floor plate (Lentz et al., 1997) and may serve to localize floor plate-derived Slit protein to the immediate vicinity of the ventral midline. Thus, while hSlit2 can act over a distance in our assay, it is possible that in vivo, in some regions in the embryo, contact-dependent Slit-mediated repulsion may predominate, which would be consistent with the contact-dependent role proposed for dSlit at the *Drosophila* midline (Kidd et al., 1999). As required by this model, we have preliminary evidence to indicate that hSlit2 can act as an inhibitory substrate for motor axons (data not shown). While the receptor mediating these actions of Slit2 remains to be determined, Robo1 and Robo2, both of which are expressed in the developing motor column, are strong candidates.

Understanding the role of Slit in patterning motor axon trajectories in vertebrates is complicated by the fact that, in contrast to what is observed in *Drosophila*, Slit2 and -3 in the rat are expressed not just in the floor plate, but also at high levels in the developing motor column. If Slit proteins are repellents for motor axons, why would these genes be expressed within the motor column, and why does this expression not abolish motor axon responsiveness to Slit proteins? One possibility is that there is selective expression of the cleaving protease, perhaps in the floor plate, and that only the floor plate-derived, processed Slit proteins can repel motor axons. Although these studies need to be extended in vivo to determine the precise role of Slit proteins in motor axon guidance, the fact that Slit2 can repel spinal motor axons is consistent with a conserved role for Slit proteins in mediating repulsive axon guidance. Further studies will also be required to determine the range of neuronal populations that can be repelled by Slit proteins. In particular, it will be critical to determine whether Slit proteins function as repellents to prevent midline recrossing by commissural axons in vertebrates, as dSlit appears to in *Drosophila*. So far, we have not been able to demonstrate a repulsive effect of hSlit2 on spinal commissural axons in culture, either when hSlit2 was presented as a point source or in a uniform concentration (unpublished observations). While this may indicate that Slit proteins do not directly function in commissural axon guidance, it is also possible that this result reflects either limitations of our culture system or perhaps to a more specific problem, such as an inappropriate regulation of Robo expression in culture.

Mammalian *Slit* and *Robo* genes are also expressed

in a variety of other regions of the embryo, including both neuronal and nonneuronal tissues (Holmes et al., 1998; Itoh et al., 1998), and Slit proteins can repel various classes of axons outside the spinal cord (Ba-Charvet et al., 1999; Li et al., 1999). Moreover, the N-terminal fragment of hSlit2 has been shown to promote the elongation and branching of DRG sensory axons, indicating that Slit proteins are bifunctional (Wang et al., 1999). Further studies in vivo will be required to determine the extent of guidance events that are regulated both by Slit-mediated repulsion and by the positive actions of Slit proteins. Likewise, at the cellular level, deciphering how Slit can elicit both positive and negative responses will require looking downstream at the signal transduction components involved in translating these effects into changes in axon behavior and growth cone motility.

Experimental Procedures

Isolation of Vertebrate Robo and Slit Homologs

rRobo2 was cloned by low stringency screening of an E13 spinal cord library with probes corresponding to *rRobo1*. Database searching revealed an EST, ab16g10.r1, with homology to the ALPS domain of *Drosophila slit*. This EST was used to probe a human fetal brain library (Stratagene) and clones for human *Slit2* were isolated and sequenced. *rSlit1* and *rSlit2* were cloned by screening this library at high stringency with fragments of cDNA clones corresponding to human EST EST05940 (GenBank accession T08049) and mouse EST mjl7g01.r1 (GenBank accession AA049991), corresponding to *Slit1*, and human EST ab16g10.r1 (GenBank accession AA488867), corresponding to *Slit2*. A fragment corresponding to *rSlit3* was isolated by nested PCR from E13 spinal cord cDNA using primers designed against sequences in mouse EST ua23e09.r1 (GenBank accession AA981600) and rat ESTs g3187052 and g3247207 (GenBank accession AA996497 and A1029381, respectively). Both *rSlit1* and *rSlit2* were pieced together from multiple overlapping partial cDNA clones. The sequences of the different Slit genes have the following GenBank accession numbers: *rSlit1*, AF133730; *hSlit2*, AF133270; and *dSlit*, AF126540.

In Situ Hybridization

In situ hybridization of rat spinal cords was carried out essentially as described in Fan and Tessier-Lavigne (1994). For each gene studied, in situ analyses were carried out with at least two independent probes, with identical results.

Generation of Myc, Fc, AP, and FLAG-Tagged Fusion Proteins

Mammalian Slit and Robo expression vectors were constructed by fusing sequences encoding rRobo1, rRobo2, hSlit2, Fc, or FLAG into the expression vector pSecTagB (Invitrogen) or pCep4 (Invitrogen), while a dRobo1 expression vector was made by inserting a modified *dRobo1* cDNA (consisting of an optimized ribosome-binding site followed by the entire dRobo1 open reading frame) into pcDNA3 (Invitrogen). N-terminally tagged dRobo-AP was constructed in the expression vector APTag4 (Cheng et al., 1995). pcDNA3-dSlit (Invitrogen) was used for expression of dSlit in mammalian cells (see Kidd et al., 1999, for construction). For expression of C-terminally AP-tagged dSlit in *Drosophila* S2 cells, fragments containing the *Drosophila slit* ORF and the human alkaline phosphatase gene were cloned into the S2 transfection vector pRmHa3 (Bunch et al., 1988). Fusion proteins were generated by transient transfection of COS or 293T cells with FuGene (Boehringer-Mannheim). Cells were conditioned for 2–3 days in OptiMEM (Gibco) and conditioned medium was concentrated on Centricon-30 microconcentrators (Amicon) when necessary. For S2 cell transfections, S2 cells were cotransfected with pRmHa3-*slit* and pPC4, and conditioned medium containing AP-dSlit was obtained following standard procedures (Bieber, 1994).

Antibodies and Western Analyses

To examine processing of hSlit2, conditioned media and high salt (1 M NaCl) extracts from cells transfected with either N-FLAG-hSlit2 or C-myc-hSlit2 expression constructs were collected, TCA precipitated, solubilized in SDS-PAGE sample buffer, run out on an SDS-PAGE (7.5%) gel, and Western blotted by standard methods with either a monoclonal antibody against the N-terminal FLAG-epitope tag (M2) or against the C-terminal myc-epitope tag (9E10; gift of J. M. Bishop). Embryo lysates prepared from 0–22 hr *Drosophila* embryos and conditioned media from S2 or mammalian cells were diluted in SDS-PAGE sample buffer, separated by SDS-PAGE, and Western blotted with a 1:20 dilution of the anti-Slit mAb C555.4C (R. Mann and S. Artavanis-Tsakonas, unpublished), which is directed against an antigen in the 7th EGF repeat region of dSlit.

Determination of the Putative hSlit2 Cleavage Site

Conditioned media from cells transfected with C-myc-hSlit2, which bears a histidine tag at its C-terminus, was concentrated using a Centrprep-30 concentrator and incubated with nickel-agarose beads, since this protein, expressed from the pSecTag vector, also bears a histidine tag. After washing, bound protein was eluted from beads with increasing concentrations of imidazole and analyzed by SDS-PAGE (7.5% gel). The gel was electroblotted to a ProBlott membrane (Applied Biosystems) and a band corresponding to the predicted size of the C-terminal fragment (60 kDa) was excised and its amino-terminal sequence was obtained by Edman degradation.

Binding Experiments

COS or 293T cells were transiently transfected as described above, and cell overlay assays carried out essentially as described (Keino-Masu et al., 1996).

Equilibrium Binding Experiments

Robo1-Fc, Robo2-Fc, and dRobo-Fc were produced as described above and purified on a Protein A-sepharose column by standard means. Netrin 1(VI-V-Fc) was purified from a stable 293-EBNA cell line. Binding on cells mock-transfected or transiently transfected with hSlit2 or dSlit was quantified essentially as described (Keino-Masu et al., 1996) except that after binding, cells were washed three times with PBS/1% BSA, fixed sequentially with methanol and 4% PFA, and washed again with PBS, before incubation with the ¹²⁵I-conjugated anti-human IgG secondary antibody. Binding of Robo1, Robo2, and dRobo was performed on COS cells. Netrin 1(VI-V-Fc) binding was measured on 293T cells (because of its high background binding to COS cells). In each experiment, concentrations were assayed in triplicate.

Motor Neuron Repulsion Assay

Dissection and culture of E11 (E0 is the day of vaginal plug) rat spinal cord was performed essentially as described (Ebens et al., 1996) except that explants and COS cell aggregates were cultured in a 50:50 mix of rat tail collagen and Matrigel (Collaborative Sciences), were supplemented with 50 ng/ml heparin sulfate, and were cultured for 30 hr. Cultures were fixed in 4% PFA and stained as described (Kennedy et al., 1994) with a monoclonal antibody against neurofilament (gift of Dr. V. Lee) and an anti-mouse IgG Cy3-conjugated secondary antibody in PBS/0.1% Triton X-100/1% heat-inactivated normal goat serum.

Assays were quantified by measuring the ratio of axon outgrowth extending from the proximal versus distal sides of the explant (with respect to the cell aggregate). Stained explant cultures were photographed and digitized on an Axiophot fluorescence microscope using a CCD camera. Representative samples from four different experiments were selected for analyses. Excluded were cultures in which explants were nonuniformly stained, lacked neurite outgrowth altogether, or were positioned either <75 μ m or >200 μ m away from the cell aggregate. Images were subdivided into proximal and distal regions, and axon outgrowth originating from proximal and distal sides was determined by measuring the area of total fluorescence associated with each region. We chose to measure outgrowth associated with halves of explants rather than quadrants because outgrowth from ventral spinal cord explants is often not radial, with less outgrowth seen on the floor plate side of the explant (which,

in all cultures, was oriented perpendicular to the cell aggregate). To avoid variability due to shape and orientation of explants, fluorescence associated with the body of the explant was excluded from the analysis. The area of outgrowth was chosen as an index of growth because counting either numbers or length of axons in these cultures would be impossible, both due to the fact that many axons are found in fasciculated bundles, making it difficult to assess numbers of neurites, and because individual axons grow in three dimensions, making it difficult to measure lengths.

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GenBank Accession Numbers

The sequences of the Slit genes reported in this paper have the following GenBank accession numbers: *rSlit1*, AF133730; *hSlit2*, AF133270; and *dSlit*, AF126540.